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NITRATE REDUCTASE STUDY FROM HEAT SUSCEPTIBLE AND HEAT TOLERANT CULTIVAR OF WHEAT (*TRITICUM AESTIVUM* L.)

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ABSTRACT

The present work leads to the study of Nitrate reductase enzyme particularly from heat tolerant and heat susceptible cultivars (*Triticum aestium* L) plants were continuously subjected to different kinds of stress at different levels and of different intensities. In the present study different cultivars of wheat (*Triticum aestivum L*) identified by plant breeders of IARI as heat tolerant or heat susceptible were used to study the process of nitrate reduction in leaves. The enzyme nitrate reductase (EC: 1.6.6.1) was studied under *in vitro* conditions. The extraction medium for the enzyme was made up of phosphate buffer (pH 7.5), EDTA and Cystein HCl. The leaf extract was centrifuged at 10000 x g, 15 min. and supernatant was used as the source of enzyme. Nitrate reductase activity from C-4 (maize) was slightly higher as compare to both heat tolerant and susceptible cultivars of wheat. Enzyme was fairly stable up to 24 hours when stored frozen (-20^{0}C). The loss of activity was only 15-20% after 48 hours. The two kinds of cultivars though have near similar Michealis Menton constant (Km) values for co-enzyme (NADH), but km for nitrate (substrate) was significantly different. The km value with heat tolerant cultivar was 9.52X10^{-3}M and for heat susceptible it was 33.3X10^{-3}M. Nitrate reductase activity decreased after the exposure of seedling to temperature of 40^{0}C in light, and major decrease in activity was during the first 30 min. Sucrose imparted protection to the enzyme in heat tolerant cultivar, when excised leaf blades were exposed to 45^{0}Cfor 30 min. Nitrate reductase (*in vitro*) from both the cultivars showed unusually higher activity against the control when Mg^{2+} ions were present in the assay medium, while EDTA showed marginal decrease in the enzyme activity. The Wheat enzyme seems to have unique property of activation by divalent cat ion (Mg^{2+}).

KEYWORDS: Wheat, Nitrate Reductase, NADH, Mg^{2+}, EDTA, Km, Line Weaver-Burk

INTRODUCTION

Nutrient requirement of the green plants is fulfilled by the soil and atmosphere. Among them nitrogen the key elements of plant nutrition no doubt occupies the foremost and imposingly distinct status for proper growth and development. Its importance in the metabolism of all organisms can readily be appreciated from the mere consideration that it is the important constituent of physiologically vital molecules as well as of informational macromolecules. Although it is the inorganic form of nitrogen being taken up by the plants, most of the soil reserve nitrogen is in the organic form (Brady, 1974). The organic and ammonium form of nitrogen is ultimately giving rise to nitrate by nitrifying bacteria. There are three main forms of nitrogen taken up by the plants, ammonium, nitrate, and di-nitrogen fixed symbiotically by

the *Rhizobium.* Legume association. But nitrate form is the most important for crop production. Thus with very few exception, most of the higher plant derive their nitrogen requirements from soil predominantly in the form of nitrate (Virtanen and Rautenen 1952, Bear 1969).

It is surprising that only within the last two decade serious attention has been directed to study the nitrate uptake by the plants. Studies on nitrate uptake have been hampered by the lack of readily available radioactive isotope of nitrogen. Nitrogen can be rapidly metabolized after uptake, so that a radioactive marker of reasonable half life fate of individual nitrogen atom in the same way as was ^{14}C in Calvin and Benson's classic experiment in which the stable $^{15}NO_3^-$ is fed to the plants tend to be time consuming and expensive, and methods of analysis of ^{15}N are comparatively insensitive (Deane- Drummond, 1990). Recently $^{36}ClO_3^-$ has been found to be suitable radioactive analogue for $^{15}NO_3^-$ in short term studies but its phyto-toxicity precludes its use in long term experiments (Deane-Drummond 1985).

Although mostly nitrate is taken up by the plants, ammonium form is ultimately utilized for the formation of organic nitrogenous compounds so the absorbed nitrogen must be reduced to form ammonia. The conversion of nitrate to ammonia by living tissue is called nitrate assimilation. It is now well established that conversion of nitrate to ammonia is catalysed by two enzymes. The first enzyme which catalyses the conversion of nitrate to nitrite is called nitrate reductase (E.C. 1.6.6.1) and the second enzyme which catalyses the conversion of nitrite to ammonia is called nitrite reductase (E.C. 1.7.7.1).

Besides the higher plants, a group of facultative anaerobes (Ex. *Pseudomonas denitrificants*) can utilize nitrate as an alternative electron acceptor in the absence of oxygen, which is known as "nitrate respiration" or "nitrate dissimilation".

Since nitrate reductase was established as rate limiting catalyst and key regulatory site in higher plant nitrate utilization (Campbell. 1990). The enzyme, which has been extensively studied from plants, fungi and microorganisms to understand the mechanism of enzyme reaction. Over the last decades when much of the biochemistry of nitrate reductase has been well understood, in addition a new aspect has been opened in the study of nitrate reductase with the introduction of antibodies specific for the higher plant enzyme (Campbell and Remmler, 1986, Cherel *et. Al.,* 1985, Notton *et. al.,*1985, Vaugh *et.al., 1984*). Regarding the location of these two enzymes (NR and NiR) it has been agreed in green tissues nitrite reductase is present in the chloroplast and utilizes photo-inducible reducing power reduced ferodoxin. The location of nitrate reductase though thought to be in cytoplasm but it has also been indicated that for its activity enzyme requires membrane association (Drummond and Johnson 1980) and utilizes NAD (P) H as reducing power.

The uptake of nitrate by plants takes place against an electrochemical gradient (Higuinbotham, 1973), suggesting the active absorption (Heimer and Filmer 1971; Higuinbotham, 1973). The uptake is regulated both at the step of its entry into the self and further into ammonical form, the nitrate assimilation process is under extensive study from different laboratories for variable reasons beside to understand the mechanism of the process and reducing power sources. This understanding will help in making genetic manipulation for increasing amounts of reduced nitrogen in the crop plants with overall enhancement of crop yield.

Evidences have accumulated in recent years that assimilatory nitrate reductase in leaves and roots can be modulated rapidly in response to environmental factors such as light CO2 or oxygen availability (Glaab and Kaiser, 1995) Various direct (Huber et. al. 1992) and indirect observation (Kaiser and Huber 1994) have shown that inactivation of nitrate reductase was caused by NR proteins phosphorylation and reactivation by de-phosphorylation. Kaiser and Spill in

1991 first presented their hypothesis that NR is reversibly phosphorylated and that phosphorylated NR is inactivated by confirmation changes due to chelation of divalent cation with the phosphoryl groups. Spinach NR protein is phoshporylated immediately after dark treatment at serine 543 which is located in the hing one region connecting the Cytochrome b domain with the molybdenum cofactors binding domain (Bachmann, et. al 1996, Sueyoshi et. al., 1998).Modulation of NR activity by light/dark transition has also be observed with the leaves of several species, including *Brasica campestris* (Kojima *et al.*,1991), pea (Glab and Kaisier, 1993), *Arabidopsis* (LaBrie and Crawford, 1994) and maize (Merlo *et al.*, 1995). Light/dark transition inactivates NR which is without changes in levels of NR protein in *Brasica campastris* (Kojima *el al.*,1991)

In the present study the use has been made of phenotypically temperature adopted wheat cultivars. The one, heat tolerant cultivar and other, heat susceptible. The enzyme nitrate reductase (protein) has been isolated using differential centrifugation. The enzyme from heat tolerant and heat susceptible cultivar has been subjected to different temperature regime to see the behaviour of th enzyme towards temperature from two kinds of cultivars. The results were expected to unravel the vulnerability, or stability of nitrate reductase subjected to exogenous temperature influences in heat tolerant and heat susceptible wheat cultivar.

MATERIALS AND METHODS

Plant

Present investigation was carried out with 15 days old heat susceptible HD-2329 and heat tolerant C-306 cultivars of wheat *Triticum aestivum* L. The seedlings were raised in the earthen pots filled with garden soil. They were periodically irrigated with 15mM potassium nitrate solution. Seeds were procured from the division of Genetics IARI, New Delhi-110012. The bio-chemicals were purchased from Sigma Chemicals Company, other chemical of regular use were purchased from Indian companies SRL, SDF, Qualigen.

Growing of Seedlings

Seeds were washed many times, first in tap water and then in double distilled water and partially dried in filter paper folds. The earthen pots were filled with soil and one fourth of farmyard manure. The seeds were sown in earthen pots. The pots were kept in the divisional net house under natural condition for the growth of seedlings. They were periodically irrigated with 15mM KNO_3 solutions.

To study the effect of thermal stress four pots of each heat susceptible and heat tolerant wheat cultivars were utilized. Two of each lot was kept at 30^0C in shade and at 45^0 C in light. After 30min leaf blades were cut and taken for the extraction of enzyme.

Enzyme Extraction

Uniformly grown healthy leaves were removed from the wheat plants, washed thoroughly with distilled water and blotted partially dried with the help of filtered papers folds and kept on ice till used. Extraction medium was composed of sodium phosphate buffer, pH 7.5, 0.2M; Na_2-EDTA, 1mM and cysteine Hydrochloride, 1mM. The leaf tissue to extraction medium ratio was 1:3 both for heat susceptible and heat tolerant cultivars of wheat. The tissue was ground in chilled mortar with pestle using extraction medium.

The resultant extract was passed through four layer of muslin cloth and centrifuged at 10000 x g for 15 minute. The supernatant was stored in clean and cold test tube and used the source of enzyme (crude preparation). All the operations were carried out in cold (0-4^{0}C).

ASSAY OF NITRATE REDUCTASE

In Vitro

The nitrate reductase activity was determined by the estimation of nitrite produced during the enzyme reaction according to Eck and Hageman (1974).

The reaction mixture in final volume of 2ml contained sodium phosphate buffer 0.2M, pH 7.5; potassium nitrate 0.2mM; NADH 0.1mM and suitable amount of enzyme preparation. The enzyme reaction was initiated by the addition of NADH. The tubes containing reaction medium were incubated at 33^{0}C for 30 minute and reaction was stopped with .2ml of 1M zinc acetate. NADH was omitted from the parallel controls to which it was added after addition of zinc acetate. The content of the tubes were thoroughly mixed and centrifuged at 3000X g for 10 minute; a suitable aliquot of supernatant was taken for estimation of nitrite.

Nitrite Estimation

After the reaction was stopped as indicated above, a suitable aliquot was taken for estimation of nitrite. To the aliquot, an amount of distilled water was added to make it 1ml. The blank tubes contained 1ml distilled water. To each tube 1ml of 1% (w/v) solution of sulphanilamide prepared in 1N HCl and 1ml of 0.02% (w/v) aqueous solution of N-1 Napthyletheline di amine dihydrchloride were added with immediate stirring of tubes, followed with 2ml of distilled water, the final volume being 5ml. The tubes were allowed t stand for 20 minutes. And the color absorbance read at 540 nm in spectrophotometer. The amount of nitrite in the unknown was calculated from the nitrite standard curve.

Enzyme Unit

One unit of enzyme activity represent one micro mole of nitrite formed per hour per gram fresh tissue under the standard assay condition.

TREATMENTS

Enzyme

In order to study the linearity of the enzyme reaction different amount of enzyme was taken in the reaction tube and enzyme activity assayed.

Nitrate

In order to study the effect of nitrate on the *in vitro* nitrate reductase, different concentration of nitrate, with 0.02mM, 0.05mM, 0.1mM, 0.2mM, 0.3mM and 0.4mM were included in the assay system of the enzyme prior to start the enzyme reaction.

NADH

In order to see the effect of NADH concentration on the *in vitro* enzyme activity different concentrations of NADH were used for initiating the enzyme reaction. NADH used was, with 0.05mM, 0.1mM, 0.2mM, 0.4mM and 0.8mM.

Sucrose

To see the effect of sucrose on *in vitro* nitrate reductase activity, the leaf blades of the both tolerant and susceptible wheat cultivars with bases dipped in sucrose solution in beaker were placed at 30^0C and 45^0 C for 30 minutes. Thereafter, the leaf blades from each lot were washed with distilled water, the enzyme extracted and *in vitro* nitrate reductase studied. In controls the sucrose solution was replaced with distilled water.

Ma^{2+} and EDTA

Heat susceptible and heat tolerant cultivar of wheat were grown as indicated earlier. Earthen pots containing 15 days old uniformly grown seedlings were placed in dark for 12 hour. Thereafter, one set of pots were kept at 45^0C and the other sets were kept at 30^0C for 30 minutes. Enzyme was extracted from the leaves and prior to use stored in cold.

Different test tubes were prepared for the enzyme assay (*in vitro*) and prior to start of the reaction, $MgCl_2$ 5mM or EDTA 10mM was incorporated in assay system to study their effect,

Light

In order to see the response of light on Nitrate reductase, again 15 days old wheat seedlings were used. The pots were kept in dark for 12 hours and prior to their transfer to light condition the leaves were taken for extraction of enzyme activity (0 time activity). The earthen pots were placed underneath fluorescent tubes (1300 Lux) and leaf sample for the extraction of enzyme were taken periodically after 12, 36 and 48 hours. The enzyme extraction was as done earlier.

Similarly to compare the effect of natural light (45000 Lux) and fluorescent tube light the 15 days old wheat seedlings were either placed underneath fluorescent tube of in sun light and leaf sampl were taken for 8 hour at the interval of two hour each.

Temperature

Earthen pots containing 15 days old seedlings of wheat cultivars, *viz* Olsen dwarf (Dwarf) and NP-799 (Tall) were placed at 40^0 C and first leaf samples were taken after 30 minute followed after the interval of 15 minutes for 90 minutes. The initial value (Zero time) was when earthen pots were at 32^0C.

Storability

In order to study the stability of the extracted Nitrate reductase, the enzyme was extracted as mentioned above and it was stored -20^0C for five days and the enzyme activity was studied at every 24 hours intervals. Zero time activity was considered as control.

Protein Estimation

The Lowry et. al.(1951) method for the estimation of the protein was used it been more sensitive as comparative to Biurate method.

1ml of extract was precipitated with equal amount of 10% Trichloroacetic acid (Final concentration of assay being 5% w/v). The tubes were kept at 4^0C over night. Next the centrifuge tubes were centrifuged at 3000x g, 10 minutes. The pellet was again treated with 5ml of trichloroacetic acid and centrifuged as above. The pellet was washed twice with acetones, first with 100% acetone and second time with 80% acetone (v/v) as explained (Singh and Krishnan, 1977).

Finely pellet was dissolved in 0.1N NaOH by placing the tubes in boiling water both for 10 minutes. The tubes were then centrifuged at 3000x g, 10 minutes to pellet non proteinous material at the bottom and the supernatant was used for estimation of protein by the method of Lowry *et. al*. (1951), with bovine serum albumin as standard.

RESULTS

Nitrate reductase was studied extensively from wheat. In some experiment nitrate reductase was also compared from heat tolerant, heat susceptible and maize leaves. Extraction of the enzyme for *in vitro* study was by subjecting the plant tissue to moderate grinding force using chilled mortar and pestle in the medium made up of the phosphate buffer, pH 7.5, Na_2-EDTA and cysteine –hydrochloride. The resultant extract after passage through four layers of muslin cloth was centrifuged at 10000x g, 15 minute. The supernatant was used as the source of the enzyme. All the operations were carried out in cold (0-4^{0}C).

Nitrate Reductase from Wheat and Maize

In vitro estimation of Nitrate reductase was explained as above. The activity was expressed in terms of nitrate produced per hour per gram fresh tissue. The enzyme activity from heat tolerant and het susceptible wheat cultivars was almost similar however; the enzyme activity from maize leaves was 13% higher as compared to wheat cultivars (Table 1).

Linear Relationship between Nitrate Reductase and Concentration of Enzyme

Nitrate reductase activity (*in vitro*) was expressed in terms of micro mole nitrite formed per hour per mg protein. The enzyme from both heat tolerant and heat susceptible wheat cultivars was estimated at different concentrations of enzyme (Table 2). The concentrations of enzyme taken were 0.01, 0.02, 0.05, 0.10, 0.20, and 0.30ml with parallel controls. The linear relationship was observed between the enzyme activity and enzyme concentration used, with both heat tolerant (C-306) and heat susceptible (HD-2329) wheat cultivars as shown in Figure 1.

Storage of Nitrate Reductase under Freezing Condition

The enzyme was isolated from fresh leaves of wheat cultivars and *in vitro* activity of nitrate reductase was immediately estimated (zero time control). Thereafter, the enzyme preparation was stored frozen in different set of 2ml polypropylene, screw cap tubes of different time periods. The enzyme was quite stable for 24 hour and there was a gradual decrease, being 20%, 36%, 79%, 84 %, with heat tolerant and 15%, 26%, 68%, 81%, with heat susceptible wheat cultivar after 48, 72, 96, and 120 hour of frozen storage, respectively (Table 3). The isolated enzyme, thus safely can be used up to 72 hours for the study of enzyme profile from one stock preparation.

Nitrate Specificity of *In Vitro* Nitrate Reductase

In this study fresh preparation of enzyme was used. At one concentration of NADH and enzyme, the concentration of Nitrate was varied from 2mM to 40mM with enzyme from both heat tolerant and heat susceptible wheat cultivars (Table 4). Enzyme from heat tolerant wheat cultivar showed gradual increase in enzyme activity up to 40mM (Figure 2), while the enzyme from heat susceptible wheat cultivar activity was slow at lower substrate concentration of 2mM and 5mM, but there after the increase was tremendous and activity at 40mM was 30% higher as compare to thFat with heat tolerant cultivars (Figure 2).

Line weaver-Burk Plot, obtained by plotting 1/[v] vs 1/[s], showed the Km values of 9.52mM and 33.3mM for substrate (nitrate) for the *in vitro* enzyme nitrate reductase from heat tolerant and heat susceptible (Figure 3) wheat cultivars.

In Vitro Nitrate Reductase at Different Concentration of NADH

Fresh enzyme preparation was used for the study. At one concentration of nitrate and enzyme, the concentration of NADH varied from 0.05mM to 0.50mM with enzyme from both heat tolerant and heat susceptible wheat cultivars (Table 5). Enzyme from heat tolerant and heat susceptible wheat cultivar showed tremendous increase in enzyme activity with the increase in concentration of NADH in the reaction mixture (Figure 4). The maximum increase in enzyme activity with tolerant and susceptible wheat cultivars was at 0.3mM and 0.4mM NADH, respectively, thereafter, decrease in both cultivars was observed (Figure 4).

Line weaver-Burk Plot, obtained by plotting 1/[v] vs 1/[s] showed the Km values of 0.83mM and 0.63mM for NADH (co-enzyme) for the in vitro enzyme nitrate reductase from heat tolerant and heat susceptible (Figure 5).

The Effect of Exposure of Wheat Seedling 40^0C

The 15 days old seedlings of wheat cultivars in earthen pots *viz.* Olesons dwarf and NP-799 were placed in light at 40^0C for varying time interval and *in vitro* nitrate reductase was estimated. Zero time extraction was taken as control, there was a drastic reduction in enzyme activity with both the cultivars after initial 30min exposure, being 45 to 49%, thereafter 45 min, 60 min, 75 min, and 90 min exposures the decrease was slow from 54 to 72% (Table 6). The results indicated that major decrease in enzyme, nitrate reductase, takes place during the first half an hour exposure of the seedling to elevated temperature, thereafter seedlings show acclimation to the elevated temperature.

Effect of Sucrose on the *In Vitro* Nitrate Reductase

The 15days old seedlings were given 12 hour dark treatment and then the leaf blades were supplied 1mM sucrose at 30^0C and 45^0C for 30 minute in light. The enzyme activity in heat tolerant and heat susceptible cultivars supplied with sucrose is similar to that when leaf blades were kept in water (Table 7). However, the enzyme activity in heat tolerant cultivar was 14% higher and in heat susceptible 15% lower at 45^0C with sucrose against water control. The results indicated that sugar imparts protection to enzyme in heat tolerant wheat seedling at elevated temperature where as it was not so with heat susceptible cultivar.

In Vitro Nitrate Reductase in Presence of Mg^{2+}, EDTA, or Mg^{2+}Plus EDTA

The wheat seedlings were placed in dark for 12 hours and then divided in to two lots one lot was kept at 30^0C and other lot at 45^0C in light for 30 minute. The enzyme activity in both heat tolerant and susceptible cultivar was higher in seedlings placed at 45^0C. In both heat tolerant and heat susceptible cultivars the effect of Mg^{2+} ions was tremendous when seedlings were exposed to high temperature of 45^0C in light the increase was 50% and 31%, respectively (Table 8). The increase in enzyme activity was also observed when seedlings were exposed to 30^0C but the degree of increase was marginal 14% (Table 8). Higher level of activation of enzyme activity in heat tolerant cultivar with Mg^{2+} ions seems due to activation of phosphatase, which renders more nitrate reductase to be dephosphorylated form.

In Vitro Nitrate Reductase in Wheat Cultivar Exposed to Room Light after 12 Hour Dark Treatment

The 15 days old seedlings were placed in dark for 12 hours and then one lot was allowed to remain in dark and the other lot was exposed to room light (approximately 1300Lux light intensity). The seedlings exposed to room light showed marginal increase of 16-18% in 36 hours exposure in both the cultivars. Whereas drastic decrease (81 %) in enzyme activity was observed in the seedlings allowed to continue in dark for 36 hours after initial 12 hours dark treatment (Table 9).

Effect of Day Light on the *In Vitro* Nitrate Reductase

The seedlings after dark treatment f 12 hours were exposed to day light (approximately 45000 Lux light intensity) for varying time intervals up to 8 hours. In both the cultivars there was significant increase in enzyme activity being 46% and 59% in heat tolerant and heat susceptible wheat cultivars, respectively (Table 10).

DISCUSSIONS

The aim of the investigation was to compare the enzyme nitrate reductase for heat tolerant and heat susceptible cultivars of wheat.

Extraction of Nitrate Reductase

Extraction medium contained cysteine HCl because the enzyme nitrate reductase in apparently known as sulphydryl containing enzyme and cysteine gives protection to sulphydryl group on the enzyme (Nicholas and Nason, 1957). Phosphate buffer was used in both extraction and assay media, because phosphate is known to stimulate this enzyme due to the existence of a phosphomolybdate complex in the enzyme which renders the enzymie molybdate much more reactive than when it is in the Free State (Kinsky and Mc Elroy 1958; Oji et.al., 1987). EDTA was present in the extraction medium to protect the enzyme from harmful effects of the metal ions present in the vacuoles, which are released during cell disruption. EDTA incorporation in the extraction medium appeared beneficial for corn and figure weed (Klepper *et. al.* 1971).

Nitrate Reductase from Wheat and Maize Leaves

The activity of maize nitrate reductase (*in vitro*) was almost similar in heat tolerant and heat susceptible cultivar of wheat but the enzyme showed slightly higher level in maize, when studied under similar condition (Table 1). Maize leaves have been reported to be 2-3 times more efficient in nitrate reduction compared to barley a C-3 plant (Dhawan and Goyal, 2004). The difference in the responses of wheat and maize to nitrogen nutrition have been related to differences in the assimilation capacity of the C-3 and C-4 photosynthetic mechanism of wheat and maize respectively, which is also related to differences in the availability of carbohydrates within the roots of these plants (Lewis *et. al.*, 1990; Cramer and Lewis, 1993). When studied the enzyme (*in vitro)* showed a linear relationship between the enzyme concentration used and enzyme activity in both kind of cultivars (Table 2). Care has always been taken the enzyme concentration in linear range *i.e*. enzyme concentration against enzyme activity.

Storability of Nitrate Reductase Enzyme

The enzyme nitrate reductase is known as labile enzyme, which gets degraded fast during storage. It was observed that nitrate reductase isolated from both heat tolerant and heat susceptible cultivars of wheat is quite stable up to 24 hours,

and after 48 hours the decrease was marginal 15-20% (Table 3). For our studies, use has been always made up of fresh isolated enzyme preparation.

Nitrate Reductase (*In Vitro*) Reaction with Different Concentration of Substrate, Nitrate

The enzyme used was partially purified (40%) by differential sedimentations. Variable Michaelis constant (Km) values have been reported from enzyme nitrate reductase from different plant sources by different research workers, 4.0 $X10^{-4}$ M from tomato leaves, 2.3 $X10^{-4}$M from tomato roots (Sanderson and Cocking, 1964), 7.5 X 10^{-4} M from soya bean leaves (Evans and Nason, 1953), wheat embryo, 3.8X 10^{-4}M (Cresswell 1961), Marrow leaves, 1.8 X 10^{-4}M (Spencer, 1959). The nitrate Km value of 7.0 X 10^{-5}M was observed with purified enzyme from squash (Campbell and Smarrellif, 1978). The Km value for purified enzyme from 2 row barley leaves was 3.1X 10^{-6} M (Oji. *et.al.*, 1987), and $4X10^{-5}$M for spinach (Kaiser and Spill 1991). In this study with crude extract as in other cases the value of 9.52 $X10^{-3}$M and 33.3 X 10^{-3}M, respectively for wheat heat tolerant and susceptible cultivar was observed (Figure 3). The results distinctly show higher efficiency of heat tolerant wheat cultivar for nitrate utilization as compared to heat susceptible.

Oji *et.al.*(1987) observed variations in Km values with purified enzyme from 2-row barley leaf and such nitrate reductase Km values, 3.1X 10^{-6}M to 5.8X 10^{-3}M suggests that optimal orientation of nitrate by the enzyme is important in the formation of the nitrate molybdenum complex.

Nitrate Reductase (In Vitro) Reaction with Different Concentration of Co-Enzyme, NADH

The variations have also been reported for the Michaelis constant (Km) for Co-enzyme NADH from different plant sources, It was $23X10^{-6}$M from tomato leaves and $6X10^{-6}$M from tomato roots (Sanderson and Cocking, 1964), $30X10^{-6}$M from soyabean leaves (Evans and Nason, 1953), $8X10^{-6}$M from wheat embryos (Spancer and Wood, 1954). The Co-enzyme (NADH) Km value of $3X10^{-6}$M was observed with purified enzyme from squash (Campbell and Smarrellif, 1978) and $7X10^{-6}$ M for spinach (Kaiser and Spill, 1991). In wheat leaves from heat tolerant and susceptible cultivars the Michaelis constant (NADH) observed were $8.3X10^{-4}$M and $6.3X10^{-4}$M, (Figure 5). The Michaelis constant observed with wheat leaf from heat tolerant and susceptible cultivars are on the higher side as compare to those reported as above. When the Michalis constant for CO-enzyme (NADH) from two kinds of cultivars are not much variable but the Michaelis constant for substrate (Nitrate) from heat tolerant cultivar is 29 % of that of heat susceptible, which shows higher efficacy of heat tolerant cultivars to make maximum use of available substrate (Nitrate) in the leaves. Nitrate reductase (*in vitro*) at 40^0C. Lag was observed in the activity of nitrate reductase when different concentration of nitrate and of NADH were present in the assay medium, the plausible explanation is could be due to in activating effect of NADH on the enzyme at lower level of nitrate as has been reported earlier for wheat leaf (Aryan *et.al.* 1983).

It was observed that maximum damage to plant nitrate assimilation process in terms of nitrate reductase level, when wheat seedlings are exposed to higher temperature of 40^0 C takes place in the first 30 minute (Table 6), thereafter loss of the enzyme activity from 30 minute 90 minutes exposure was marginal.

Beavers and Hageman (1969) pointed out that the activity of nitrate reductase in higher plant depends on environmental factors such high light intensity, CO_2 concentration, temperature, water supply and nitrate concentration. Wild and Zerbe (1977) found that the absolute nitrate reductase activity was higher under strong then under weak light condition. Such factors determine the seasonal variations in nitrate reductase activity.

Nitrate Reductase (In Vitro) in Light at Elevated Temperature

A short term exposure of wheat seedlings 10 minute at 36, 40 and 44^0C, increased the ability of nitrate reductase to re-activate after heat damage (Lyutova and Kamentseva, 2001). Heat treatment (heat hardening –hyperthermia) increase the stability of nitrate reductase against the number of inactivating factors (heating, proteolysis, *in vitro* and in *in vivo* enzyme degradation) and enhanced the ability of the plant to repair damage induced by heating. In this study sucrose seems to have marginal protective effect on the enzyme activity in heat tolerant cultivar (Table 7). Sugars also appeared to supply the energy requirement for induction of nitrate reductase under aerobic condition by substituting the light requirement (Aslam *et.al.*,1973, Travis *et. al.*,1970).

Light treatment after the plant was kept in dark for 12 hours or 24 hours, as invariably increased the enzyme activity (Table 8, 9, 10). According to Beavers *et.al.* (1965) light increases the uptake by leaves, which leads to higher intra cellular concentration of NO_3 for induction. There are evidences also there light supplies energy for maintenance of active poly-ribosomes levels, which affect the capacity for protein synthesis (Stulen, 1974, Travis *et.al.,* 1970). It appears that light probably has affected increased uptake by leaves (Beavers *et. al.*,1965), because excised leaves did not show any increase in enzyme activity when placed in light (table 7), where as whole seedlings when placed in light showed 46- 59% increase in enzyme activity. In earlier studies exposure of wheat and sorghum etiolated seedlings after 1 hour dark treatment showed more than two fold increase in nitrate reductase (*in vivo*) after 3 hours of light exposure (Manna, 1991). A substantial increase in Nitrate reductase level has been also observed in Barley seedlings up on moving them from dark to light (Melzer *et. al.,* 1989). Activation of nitrate reductase in light could also be due to oxidation of the enzyme by light as has been reported earlier also for wheat leaf (Aryan *et.al.,* 1983).

Modulation of Nitrate Reductase

Spinach leaf nitrate reductase is rapidly inactivated by serine phophorylation and subsequent binding to 14-3-3 protein in the presence of divalent cations (Kandlbinder *et.al.* 2000). Nitrate reductase activity in the presence of Mg^{2+} usually reflects activity of dephospho-nitrate reductase. In the presence of EDTA all the forms of Nitrate reductase are active and the activity measure reflects the total amount of nitrate reductase. In leaves the nitrate reductase activation state as usually 1.5-3 times higher in the light then in the dark and this behavier has been observed for a number of higher plant species belonging to different genera (Kandlbinder *et.al.,* 2000). There are significant increase in nitrate reductase activity in presence of Mg^{2+} ions in the assay medium in light at 45^0C with both heat tolerant and heat susceptible wheat cultivar (Table 8), though in dark the increase in presence of Mg^{2+} was marginal (14%). EDTA used at the concentration of 10mM instead of showing increase as in the case of spinach (Kandlbinder et.al. 2000) showed a consistent decrease with both kind of cultivar in light or dark. Further addition of EDTA along with Mg^{2+} did not alter the enzyme activity. Activation by Mg^{2+} showed that in these cultivar dephospho-nitrate reductase is predominant as compared to phospho- forms (inactive).

The enzyme nitrate reductase exhibits considerable fluctuation in *in vitro* activity in response to light to dark transition (Sihagh *et.al.,* 1979). In contrast to the higher activity in presence of excess of EDTA (20mM), as such measured activity reflects the total amount of nitrate reductase (Kandlbinder *et.al.*,2000), in our studies EDTA has decreased the *in vitro* nitrate reductase, which appeared to be unique feature of *in vitro* nitrate reductase from wheat cultivars as compared to that of spinach. Kaiser and Spill (1991) first presented their hypothesis that nitrate reductase is inactivated by conformational changes due to chelation of divalent cation with phosphoryl group (Sueyoshi *et.al.,* 1998). Spinach nitrate reductase proteinase phosphorylated immediately after dark treatment at serine -543, which locates in the hinge-1 region

connecting the Cytochrome b domain with the molybdenum Co-factor binding domain (Bachmann *et.al.*,1996). At this point, prior to interaction phosphorylated nitrate reductase with Nitrate reductase inhibitor protein (NIP) in the presence of divalent cations Mg^{2+} or Ca^{2+}, the phosphorylated Nitrate reductase is still active. Results with wheat cultivars indicates but do not necessarily prove that there may not be an interaction taking place between phosphorylated Nitrate reductase and NIP and hence, wheat nitrate reductase remained active ever in presence of Mg^{2+} ions.

CONCLUSIONS

In conclusion the nitrate reductase from wheat cultivars (heat tolerant and heat susceptible) has shown differential substrate Km values for two kinds of cultivar being $9.52X10^{-3}$M for heat tolerant and $33.3X10^{-3}$M for heat susceptible, in contrast to inhibition of enzyme by Mg^{2+} ions the enzyme from wheat is significantly activated by Mg^{2+} and appreciably stable for 24 hours at -20^{0}C.

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APPENDICES

Table 1: In Vitro Nitrate Reductase from Wheat and Maize. 15 Day Old Seedlings of Wheat Cultivars (C-306 and HD-2329) and Maize Were Used. Other Details are as in above

Cultivars	µMole NO^-_2 Produced Hr^{-1} g Fresh $Wt.^{-1}$
C-306 (Tolerant)	1.49±0.19
HD-2329 (Susceptible)	1.44±0.21
Maize	1.69±0.72

Table 2: In Vitro Nitrate Reductase Activity, Linear Relationship with Concentration of Enzyme. 15 Day Old Seedlings of Wheat Cultivars C-306 (Heat Tolerant), HD-2329 (Heat Susceptible) were Used. Data is Averages of Two Experiments

Enzyme (Ml)	µMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$	
	C-306 (Tolerant)	HD-2329 (Susceptible)
Nil	0.00	0.00
0.01	0.34	2.12
0.02	3.79	2.47

Table 2: Contd.,		
0.05	10.33	13.40
0.10	35.13	57.48
0.20	46.85	60.65
0.30	65.45	68.41

Table 3: Effect of Storage (Frozen) on In Vitro Nitrate Reductase Activity in Wheat Cultivars. Enzyme was Extracted and Kept Frozen at Minus 20^0C. The Enzyme Activity was Analysed at Different Time Intervals

Torage (Hr)	µMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$			
	C-306	% Activity	HD-2329	% Activity
Control	1.752	100	1.724	100
24:00	1.624	92.7	1.654	95.94
48:00	1.392	79.5	1.462	84.81
72:00	1.114	63.6	1.268	73.54
96:00	0.370	21.1	0.551	31.99
120:00	0.278	15.9	0.331	19.20

Table 4: In Vitro Nitrate Reductase in Wheat Cultivar at Different Concentration of Nitrate

	Nitrate		µMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$	
	[S] (Mm)	1/[S]	Activity [V]	1/[V]
C-306 (Tolerant)	Nil	0	0.00	0
	2	0.5	6.15	0.162
	5	0.2	12.31	0.082
	10	0.1	15.38	0.065
	20	0.05	21.53	0.046
	30	0.03	24.61	0.040
	40	0.025	27.00	0.037
HD-2329 (Susceptible)	Nil	0	0.00	0
	2	0.5	2.45	0.408
	5	0.2	10.57	0.095
	10	0.1	12.20	0.082
	20	0.05	19.51	0.051
	30	0.03	28.44	0.035
	40	0.025	34.94	0.028

Table 5: In Vitro Nitrate Reductase in Wheat Cultivar at Different Concentration of NADH

	NADH (Mm)		µMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$	
	[F] (Mm)	1/[F]	Activity [V]	1/[V]
C-306 (Tolerant)	Nil	0.00	0.00	0
	0.05	20	5.02	0.199
	0.10	10	18.41	0.054
	0.20	5	33.48	0.023
	0.30	3.3	56.91	0.017
	0.40	2.5	45.20	0.022
	0.50	2	41.85	0.024
HD-2329 (Susceptible)	Nil	0	0.00	0.00
	0.05	20	3.65	0.274
	0.10	10	12.77	0.078
	0.20	5	29.20	0.034

	Table 5: Contd.,			
	0.30	3.3	38.32	0.026
	0.40	2.5	45.62	0.022
	0.50	2	41.97	0.024

Table 6: Effect of 40^{0}C Temperature on the In Vitro Nitrate Reductase from Wheat Cultivars. Earthen Pots Containing 15 Days Old Seedlings of Wheat Cultivars, Viz Olesons Dwarf (Dwarf) and NP-799 (Tall) were Placed at 40^{0}C. First Leaf Sample Were Taken after 30 Minutes Followed after the Interval of 15 Minute for 90 Minute. The Initial Valve (Zero Time) was When Earthen Pots Were at 32^{0}C

Time (Minutes)	mMole No^-_2 Produced Hr^{-1}g Fresh Wt $^{-1}$	
	Dwarf (Olesons Dwarf)	Tall (NP-799)
Nil	4.37	4.52
30	2.21	2.50
45	1.74	2.06
60	1.58	1.77
75	1.28	1.30
90	1.24	1.37

Table 7: Effect of Sucrose on the In Vitro Nitrate Reductase Activity at 30^{0}C and 45^{0}C. Earthen Pots Containing Wheat Cultivars C-306 (Heat Tolerant) and HD-2329 (Heat Susceptible) were Placed in Dark for 12 Hours Thereafter, the Leaf Blades with Bases Dipped in The Sucrose 1mm or Water (Control) were Placed in the Light at the 30^{0}C and 45^{0}C for 30 Minute. The Enzyme was Extracted and In Vitro Nitrate Reductase Estimated

Treatment	µMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$	
	C-306 (Tolerant)	HD-2329 (Susceptible)
Control	0.56	0.52
30^{0}C	0.59	0.52
45^{0}C	0.64	0.44

Table 8: Effect Mg^{2+} and EDTA on In Vitro Nitrate Reductase Activity of Heat Tolerant (C-306) and Heat Susceptible (HD-2329) Cultivars of Wheat under Light and Dark Condition. Earthen Pots Containing 15 Days Old Uniformly Grown Seedlings were Placed in Dark for 12 Hours. Thereafter, One Set of Pots Were Kept at 45^0 C and the Other Set was Allowed to Continue in Dark at 30^{0}C for 30 Minute. The Enzyme was Extracted and In Vitro Nitrate Reductase in the Dark at 30^{0}C for 30 Minute. The Enzyme was extracted and In Vitro Nitrate Reductase was Estimated

Treatment		µMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$	
		Dark	Light
C-306 (Tolerant)	Control	4.80	5.49
	$MgCl_2$	5.49	8.23
	EDTA	3.43	4.46
	$MgCl_2$ +EDTA	4.46	5.14
HD-2329 (Susceptible)	Control	3.77	4.46
	$MgCl_2$	5.49	5.83
	EDTA	3.43	4.11
	$MgCl_2$ +EDTA	3.43	4.46

Table 9: In Vitro Nitrate Reductase Activity in Wheat Cultivar Exposed to Light after 24 Hour Dark treatment the Plants Were Initially Kept in Dark for 12 Hours. There after One Lot of Plants were Allowed to Stay in Dark and the Other Lot was Exposed to Room Light (1300 Lux) Control Value is for Enzyme Extracted Immediately after 12 Hours Initial Dark Treatment. The Enzyme was Extracted at Different Interval and Activity Determined

C-306 (Tolerant)		μMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$Light Exposure (Hr)			
		Control	12:00	24:00	36:00
	Room light	1.67	1.71	1.79	1.94
	Dark	1.64	1.02	0.64	0.30
Hd-2329 (Susceptible)					
	Room light	1.49	1.53	1.60	1.76
	Dark	1.47	0.94	0.64	0.28

Table 10: Effect of Day Light on In Vitro Nitrate Reductase in Different Cultivars of Wheat. The Plant were Kept in Dark for 12 Hours. There after Plant were Exposed to Day Light (45000 Lux). The Enzyme was Extracted at Different Intervals of Time and In Vitro Nitrate Reductase was Analysed as in the Test. Zero Time Activity (Control) Was after Initial 12 Hours in the Dark

Time (Hrs)		μMole NO^-_2 Produced Hr^{-1}mg Protein $^{-1}$ Natural Light (Hr)
C-306 (Tolerant)		
	Control	1.97
	2:00	1.8
	4:00	2.57
	6:00	2.69
	8:00	2.87
Hd-2329 (Susceptible)		
	Control	1.74
	2:00	1.79
	4:00	2.42
	6:00	2.55
	8:00	2.76

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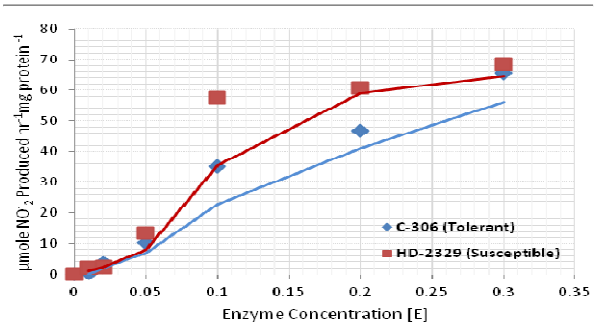

Figure 1: In Vitro Nitrate Reductase Activity of Heat Tolerant (C-306) and Heat Susceptible (HD-2329) Cultivar of Wheat, Linear Relationship with Concentration F Enzyme

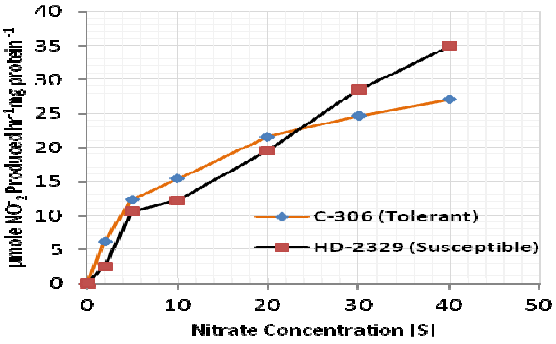

Figure 2: Effect of Nitrate on Nitrate Reductase of Heat Tolerant (C-306) and Heat Susceptible (HD-2329) Cultivar of Wheat

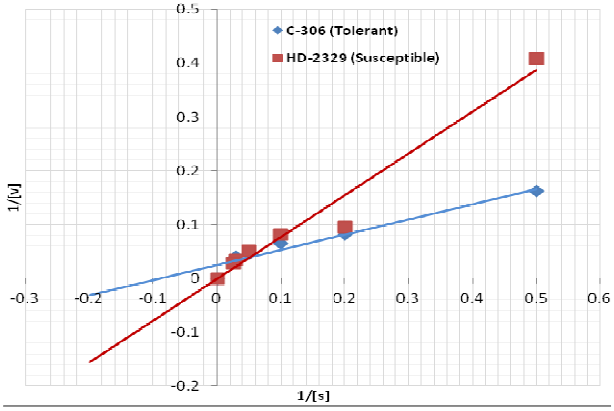

Figure 3: Line-Weavers Burk Plot of Nitrate Effect on Nitrate Reductase of Heat Tolerant (C-306) and Heat Susceptible (HD-2329) Cultivar of Wheat

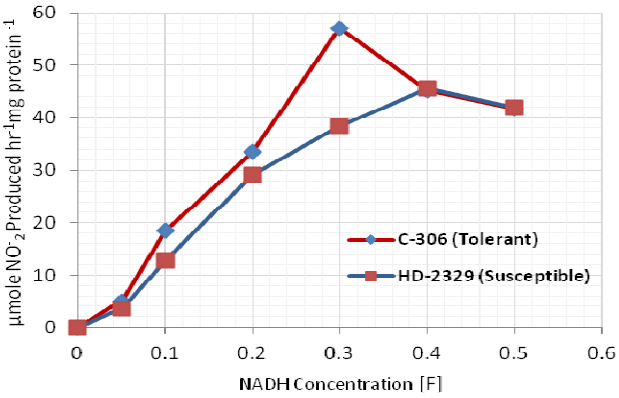

Figure 4: In Vitro Nitrate Reductase Activity in Wheat Cultivar at Different Concentration of NADH

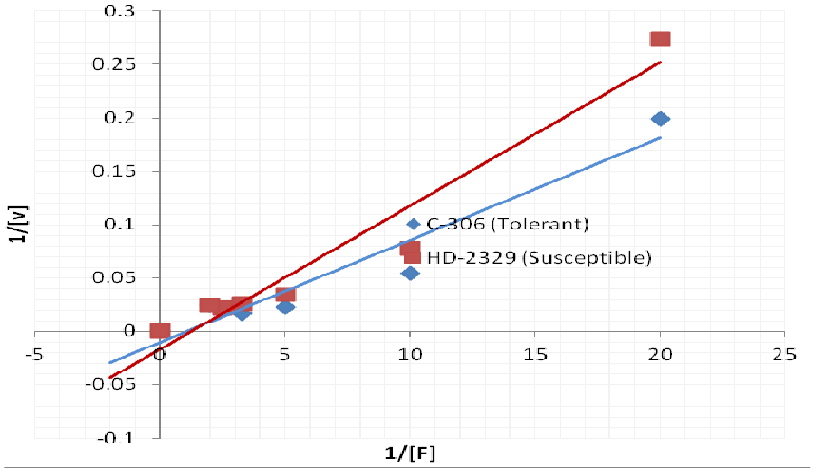

Figure 5: Line-Weaver Burk Plot of In Vitro Nitrate Reductase Activity in Wheat Cultivar at Different Concentration of NADH